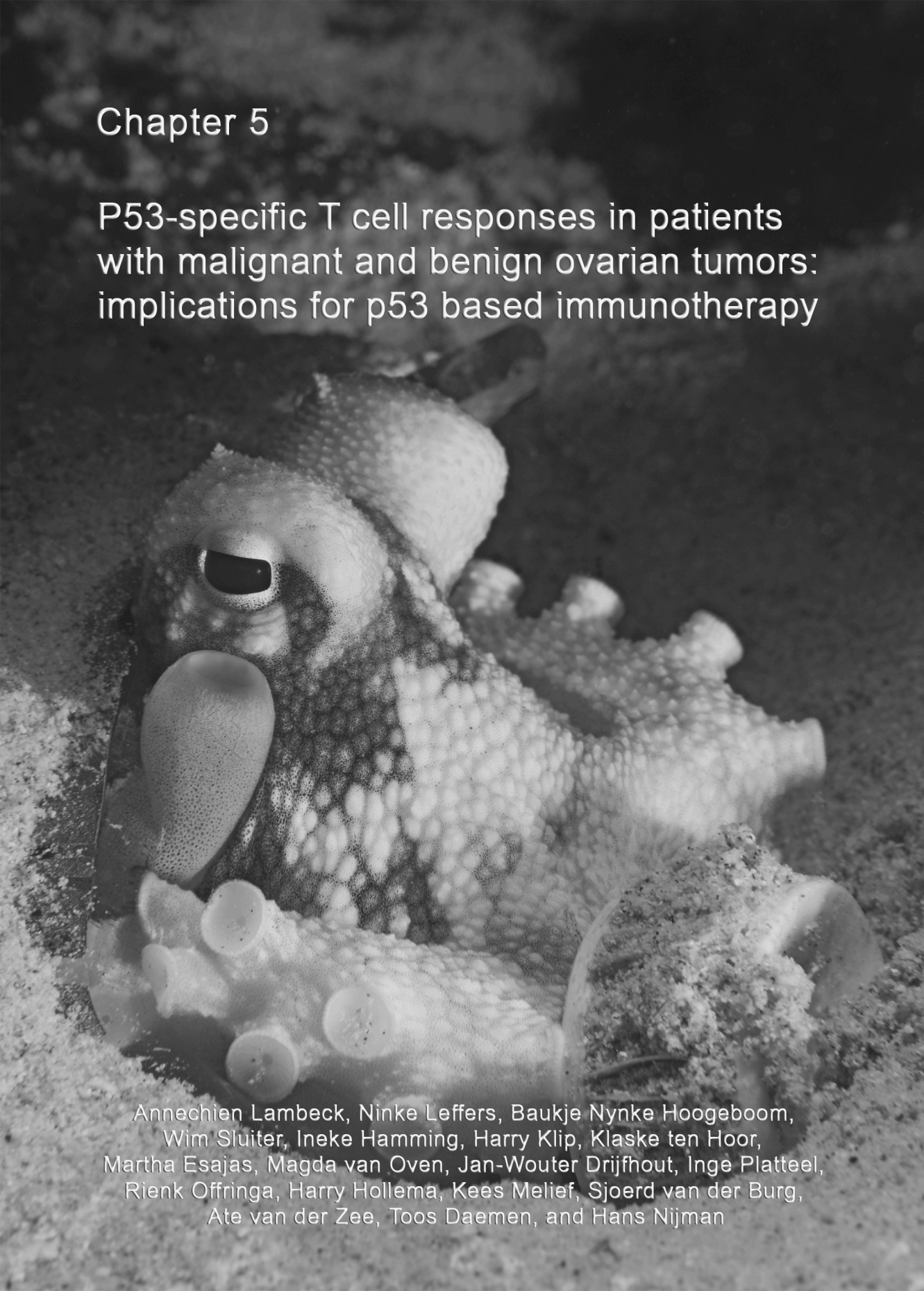
Chapter 5

P53-specific T cell responses in patients with malignant and benign ovarian tumors: implications for p53 based immunotherapy



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Abstract

Despite intensive treatment, 70% of the ovarian cancer patients will develop recurrent disease, emphasizing the need for new approaches such as immunotherapy. A promising antigenic target for immunotherapy in ovarian cancer is the frequently overexpressed p53 protein. The aim of the study was to evaluate the nature and magnitude of the baseline anti-p53 immune response in ovarian cancer patients. P53-specific T cell responses were detected in both half of the ovarian cancer patients as in the group of control subjects, consisting of women with benign ovarian tumors and healthy controls. Importantly, while in the control group p53-specific immunity was detected among the $CD45RA^+$ naïve subset of T cells only, the p53-specific T cell responses in ovarian cancer patients were also present in the $CD45RO^+$ memory T cell subset, suggesting that in the cancer patients sufficient amounts of cancer-derived p53 was presented to induce the formation of a p53-specific memory T cell response. Further characterization of the p53-specific memory T cell responses revealed that in addition to the type 1 cytokine IFN-γ also the type 2 cytokines IL-4 and IL-5, as well as the immunosuppressive cytokine IL-10 were produced. Notably, p53-specific T cells were not only detected in the peripheral blood, but also among tumor infiltrating lymphocytes and in tumor-draining lymph nodes. In conclusion, the existence of a weak mixed T-helper type 1 and 2 p53-specific T cell repertoire supports the rationale of using p53 long peptides in vaccination strategies aiming at the induction of p53-specific Th1/CTL immunity.

Introduction

Ovarian cancer is the most frequent cause of death among gynecologic malignancies. It represents the fifth leading cause of death from all cancers in women. Despite standard treatment compromising cytoreductive surgery followed by platinum/paclitaxel based chemotherapy, the overall survival for ovarian cancer patients is only 35%. In the majority of patients presenting with advanced stage disease, residual or recurrent disease will manifest itself and resistance to chemotherapy will prohibit further curative therapy (1). Therefore, new therapeutic strategies are needed to prevent recurrent disease.

The presence of intratumoral T cells is an independent prognostic marker for improved clinical outcome in ovarian cancer, demonstrating the importance of the immune system in this disease (2). In ovarian cancer, the overexpressed self-antigens Her-2 (3), NY-ESO-1 (4), SART-1 (5), Muc-1 (6), Sperm Protein 17 (7) and CA-125 (8) have been described to induce T cell responses. Another attractive target antigen in ovarian cancer is the self-antigen p53 (9;10). P53, a tumor suppressor protein, is upregulated upon DNA damage, causing cell cycle arrest and DNA repair or apoptosis. Comparable to most other malignancies, in ovarian cancer p53 is a frequently overexpressed self-antigen (50%), mostly due to mutations (11-16). The aberrant expression of the p53 protein in tumor cells versus the normal expression in nontumor cells provides an immunological window for the use of p53 as a tumor antigen for immunotherapy (10).

Since p53 is a self-antigen expressed at low levels in normal cells, immunologic tolerance might hinder the use of wild type (wt) p53 as a tumor antigen for immunotherapeutic approaches. However, preclinical data have shown that p53-specific CTL and Th cells can be induced in mice with the capacity to eradicate p53-overexpressing tumors, without inducing autoimmunity (17;18). Furthermore, p53-specific T cells against naturally processed MHC I- and MHC II-restricted p53 epitopes can be isolated from healthy donors and cancer patients (19-31). In ovarian cancer patients, p53-specific antibodies can be detected, indicating the presence of p53-specific T-helper cells (32-36). The presence of cellular and humoral immune responses against p53 shows that tolerance is not complete for this self antigen.

An extensive analysis of the nature and magnitude of the baseline cellular anti p53 immune response has not been performed in ovarian cancer patients. Aim of the study was to analyze the baseline T cell activity against p53 during standard treatment for ovarian cancer, using IFN-γ ELISPOT and proliferation assay. P53-specific T cell responses were analyzed by using long overlapping p53 peptides encompassing the whole p53 protein,

which allows the detection of HLA class I- and II-restricted p53-specific T cells irrespective of the HLA type of the patient. Our data show that low levels of activated p53-specific T cell reactivity can be found in ovarian cancer patients, indicating that p53 serves as a target antigen for the immune system and thus may be a suitable target for immunotherapy against ovarian cancer. The results of this study provide background knowledge of p53-specific T cell reactivity in cancer patients for the rational design and monitoring of p53-based immunotherapeutic vaccination strategies for ovarian cancer.

Materials and methods

Patients

Patients from the University Medical Center Groningen and the Martini Hospital Groningen were included after written informed consent. The study protocol was approved by the ethical committee of both hospitals. Forty-five patients diagnosed with primary epithelial ovarian cancer were included as well as 19 patients with benign ovarian tumors and 10 healthy women as controls. Peripheral blood mononuclear cells (PBMC) and serum were collected before and after surgery and in case of malignancy also 3 months after standard platinum based chemotherapy. PBMC were isolated from heparinized blood by Ficoll-Paque (Amersham Biosciences, Sweden) density centrifugation and frozen in RPMI (Gibco, Paisley, UK) containing 10% FCS (Bodinco, Alkmaar, the Netherlands) and 5% DMSO in liquid nitrogen until use. Serum was isolated from clotted blood and stored at -80°C until use. Tumor material was collected and partially snap-frozen, partially paraffin embedded. Tumors were evaluated for stage and differentiation grade. Data concerning age, duration of surgery, blood loss during surgery and debulking were collected. Patients with other malignancies, severe co-morbidity or on high doses of immunosuppressive agents were excluded from this study.

Immunohistochemistry of p53

Primary tumors were evaluated for p53 expression. Sections (4 μm) of paraffin-embedded tissue blocks from the primary tumors were mounted on APES-coated slides. Tissue sections were deparaffinized 2 times 10 min in xylene and rehydrated. Antigen retrieval was performed by boiling in TRIS EDTA (pH 9.0) for 8 min in a microwave oven. After 10 min cooling, the tissue sections were washed in PBS and endogenous peroxidase was blocked with peroxidase blocking reagent (DAKO, Cambridgeshire, UK) for 10 min in the

DAKO autostainer. The tissue sections were washed in PBS and incubated with DO-7 (anti-p53, 1:1,000 in PBS 1% BSA, DAKO) for 60 min, washed again with PBS 0.1% Tween and incubated with a goat anti-mouse secondary antibody conjugated with a peroxidase labeled polymer (DAKO EnVision+) for 30 min. Sections were washed and developed in liquid DAB+ substrate chromogen system (DAKO) for 10 min. The sections were counter-stained with hematoxilin, dehydrated, cleared in xylene, and mounted. The following scoring system was used for the p53 immunostaining: 0 = negative; 1 = 1% to 25% cells with p53 protein accumulation; 2 = 26% to 75% and 3 = >75% cells with p53 protein accumulation. The scores 2 and 3 were considered as p53 overexpression. Scoring was performed independently by an investigator and a pathologist (K.A.H and H.H).

Detection of serum anti p53-antibodies

The p53-specific IgG titers were quantified in the serum of the ovarian cancer patients, before and after surgery and 3 months after chemotherapy, using a commercially available p53-ELISA kit (Dianova, Hamburg, Germany), following manufacturer's instruction. The anti-p53 IgG titers (U/ml) of each serum sample were calculated at 100X dilution using a linear regression curve obtained by plotting the standard concentration versus the absorbance. Results were interpreted as follows: negative, <80 U/ml and positive, ≥80 U/ml, as indicated by the manufacturer.

Antigens

30-mer peptides overlapping by 14 amino acids, spanning the wt p53 protein, were synthesized. These 24 peptides were divided into three pools, pool 1: peptides 1-8 (residues 1-142), pool 2: peptides 9-16 (residues 129-270) and pool 3: peptides 17-24 (residues 257-393). MRM, a mixture of tetanus toxoid (150 limus flocculentius/ml; National Institute of Public Health and the Environment, Bilthoven, the Netherlands), *Mycobacterium tuberculosis* sonicate (2,5 μg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, the Netherlands) and *Candida albicans* (0,005% HAL Allergenen Lab, Haarlem, the Netherlands) was used to control the capacity of PBMC to proliferate in response to typical recall antigens.

IFN-γ ELISPOT assay

An IFN-γ ELISPOT was used to measure p53 peptide specific responses in PBMC, because of high sensitivity and the ability to use cryopreserved PBMC. Cryopreserved

PBMC were thawed and cultured at a density of 1.5-2.0x10^6 cells/well in a 24-well plate in Iscove's medium (Gibco) containing 10% autologous serum, penicillin, streptomycin and β-mercapto-ethanol. PBMC were stimulated with p1-p8, p9-p16, p17-p24 (15 μg/peptide/ml) or MRM at 1:50 dilution. After 4 days of incubation at 37°C, PBMC were harvested and cultured in Iscove's medium containing 10% FCS at a density of 10^5 cells/well in a 96-well ELISPOT plate (Nunc, Silent screen, Rochester, NY, USA) precoated with antihuman IFN-γ (Sanquin, Leiden, the Netherlands). PBMC from a patient before and after surgery and after chemotherapy were analyzed in quatro in the same ELISPOT plate. After overnight incubation the spots were visualized through incubation with biotinylated antihuman IFN-γ, HRP-streptavidin and TMB substrate (Sanquin, Leiden, the Netherlands) following manufacturer's instructions. Spots were counted with an A.EL.VIS spot analyzer (Sanquin, Leiden, the Netherlands). A patient was considered to have a p53-specific T cell response if the mean number of spots exceeded the mean number of spots of the medium + 2 x SD by 10 spots.

CD45RA depletion

PBMC were thawed and CD45RA cells were isolated with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer's instructions. Whole PBMC, isolated $CD45RA^+$ and $CD45RA^-$ populations were cultured and analyzed for p53-specific responses with an IFN-γ ELISPOT as described earlier. To the $CD45RA^+$ and $CD45RA^-$ population, 0.5 X 10^6 irradiated autologous PBMC were added during culturing.

P53-specific proliferation assay

In addition to the IFN-γ ELISPOT assay, p53-specific responses were also measured by proliferation assay. Proliferation assays enable further characterization of responses by measuring cytokine profiles in the supernatant. Freshly isolated PBMC were cultured in the same way as described above for the ELISPOT assay. PBMC were stimulated with p53 peptides for 9 days. Subsequently, cells were harvested, counted and cultured at a density of 2 X 10^5 cells/well in a 24-well tissue culture plate in 1 ml Iscove's medium containing 10% autologous serum. To each well 1 X 10^6 irradiated autologous PBMC and 2 μg/ml PHA (Remel, Lenexa, USA) were added in 0.5 ml Iscove's medium containing 10% autologous serum. After 10 days of culturing, the cells were harvested, cultured at a density of 2 X 10^4 cells/well in a 96-well microtiter plate and restimulated with the pools of p53 peptides (3 μg/ml/peptide) and 5 X 10^4 irradiated autologous PBMC in Iscove's

medium containing 10% human AB serum (Sigma). The cells were also restimulated against 12 pools containing 2 p53 peptides (p1-p2 to p23-p24) at 3 μg/ml/peptide. After 48 hr of incubation, supernatant was harvested and replicate wells were pooled and stored at -20°C for use in the cytokine bead array. Cell cultures were pulsed with 0.5 μCi [^{3}H] thymidine (MP Biomedicals) per well for 18 hr. Plates were harvested with a Micro-cell harvester (Skatron, Lier, Norway). Filters were packed in plastic bags containing 3 ml of scintillation fluid and counted in a MicroBeta counter (Wallac, Turku, Finland). The stimulation index (SI) of each culture was calculated by dividing the mean of p53-induced proliferation by the mean of the medium control. Cultures were considered positive when the SI of antigen-induced proliferation was ≥3 and ^{3}H-thymidine incorporation exceeded 10^3 counts/min. Cultures stimulated with the pools of 2 peptides contain a lower number of peptide-epitopes to which T cells can respond than cultures stimulated with the pools of 8 peptides, resulting in a decrease of sensitivity. Therefore, the reactivity of PBMC stimulated with pools of 2 peptides was considered positive when the SI of antigen-induced proliferation was ≥2 and ^{3}H-thymidine incorporation exceeded 10^3 counts/min.

Cytokine bead array

To determine the level of secreted cytokines in the supernatants of the proliferation assays of ovarian cancer patients with a p53-specific response, the production of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α was measured by cytokine bead array (LINCO*plex* kit, Linco Research, St.Charles, MO) according to manufacturer's instructions. The samples were analyzed using the Bioplex system (Bio-Rad Laboratories, Hercules, CA). Data were analyzed using a five-parametric-curve fitting. Cultures were defined to specifically produce cytokines when the amount of secreted cytokine of peptide-stimulated PBMC exceeded twice the medium control and was at least 200 pg/ml for IFN-γ and 10 pg/ml for the other cytokines.

Isolation of tumor and lymph node derived lymphocytes

Primary ovarian tumor tissue was obtained from 3 ovarian cancer patients. Tumor draining lymph nodes were obtained from 4 ovarian cancer patients. Single cell suspension from tumor tissue and lymph nodes were obtained by manually cutting the tissue followed by mashing through a cell strainer. Lymphocytes were isolated from the tumor by Ficoll-Paque (Amersham Biosciences, Sweden). Cells from tumor and lymph nodes were washed with Iscove's medium and resuspended in Iscove's medium containing 10% human AB serum

(Sigma), 5 ng/ml IL-7, 5 ng/ml IL-15 (Prepotech) and 5% TCGF (Zepto Metrix). P53-specific proliferation was measured as described above.

Statistical analysis

A Kruskal-Wallis test was performed to analyze differences in p53-specific responses before surgery between patient groups as measured by IFN-γ ELISPOT. Differences in p53-specific or MRM-specific responses after surgery between patients with malignant and benign tumors were analyzed with a Mann-Whitney *U* test. Differences in p53-specific responses before surgery, after surgery and after chemotherapy and differences in responses between the 3 peptide pools were determined by a Friedmann test. Subsequently, differences between time points and peptide pools were further analyzed with a Wilcoxon Signed Rank test. Statistical analysis of the p53-pecific responses in relation to p53 overexpression in the tumor was done by a Spearmann Rank test. Statistical significance was defined as $P < 0.05$. SPSS (SPSS 1202, Inc., Chicago, IL) was used for all analyses.

Results

Study population

Fourty-five patients with primary ovarian cancer, 19 patients with benign ovarian tumors and 10 healthy controls were included in this study. The median age of the ovarian cancer patients and patients with benign ovarian tumors was 65 yr (range, 42-84 yr) and 58 yr (range, 31-81 yr), respectively. Median duration of surgery was 189 min (range, 50-395 min) and 110 min (range, 50-170 min) for ovarian cancer patients and patients with benign tumors, respectively ($P = 0.005$). Ovarian cancer patients had significant more blood loss (median 700 ml, range, 0-4,500 ml) during surgery than patients with benign tumors (median 100 ml, range, 0-1,500 ml) ($P = 0.004$).

Twenty-three primary ovarian cancer patients (P1-P23) were analyzed for p53-specific immune responses by IFN-γ ELISPOT. Thirteen patients with benign ovarian tumors (B1-B13) and 10 healthy controls (H1-H10) were analyzed as control groups. P53-specific immune responses were analyzed by proliferation assay in 22 ovarian cancer patients (P24-P45), 9 patients with benign tumors (B10, B12-B19) and 8 healthy controls (H1-H8). ELISPOT and proliferation assays could not be done with PBMC from the same patient, benign ovarian tumor patients.

Patients with malignant and benign ovarian tumors, analyzed for p53-specific responses in the IFN-γ ELISPOT, were subsequently analyzed for serum p53-specific antibodies and p53 overexpression in the tumor (Table 1). P53 was overexpressed in 65% of the tumors of ovarian cancer patients, whereas no p53 overexpression was seen in the benign tumors. P53-specific antibodies were detected in 9% of the ovarian cancer patients and in none of the patients with benign ovarian tumors.

Table 1. Cellular and humoral anti p53 immune responses and p53 expression in the tumor in primary ovarian cancer patients

Patient[1]	p53-specific T cell responses[2]	p53-specific antibodies[3]	p53 expression[4]
P1	-	-	0
P2	+	-	3
P3	-	-	na
P4	+	-	1
P5	-	-	na
P6	-	-	2
P7	+	-	1-0
P8	na	-	3
P9	+	-	3
P10	na	-	0
P11	-	-	3
P12	+	-	3
P13	-	-	0-1
P14	-	9183	3
P15	-	-	2
P16	+	-	3
P17	+	-	3
P18	+	-	0-1
P19	-	147	0
P20	+	-	3-2
P21	-	-	na
P22	+	-	3
P23	na	-	3

[1]Twenty-three ovarian cancer patients were analysed for p53-specific immune responses, p53 expression in the tumor and p53-specific antibodies. [2]P53-specific responses before surgery, as measured by IFN-γ ELISPOT. -, no p53-specific T cell reactivity; +, p53-specific T cell reactivity. Na, not available. [3]P53 serum IgG titers (U/ml) as measured by quantitative ELISA. -, no serum p53-specific antibodies. [4]p53 expression in the tumor analyzed by immunohistochemistry with the p53-specific antibody DO-7. 0, no staining; 1, 1-25% cells with p53 protein accumulation; 2, 26-75% and 3, >75% cells with p53 protein accumulation.

P53-specific T cell reactivity in ovarian cancer patients analyzed by IFN-γ ELISPOT

In ovarian cancer patients, p53-specific responses against one or more p53 peptide pools were detected in 50% (10/20) of the patients before surgery, 9% (2/23) after surgery and 44% (8/18) after chemotherapy (Fig. 1 and Table 2). In light of the current view on T cell

tolerance to p53, the measured p53-specific responses were most likely mediated by $CD4^+$ T cells (10); however p53-specific $CD8^+$ T cells have been reported in other cancers and as such may also contribute to the response observed (37). Paired testing showed that responses before surgery were significant higher than after surgery ($P < 0.001$). P53-specific immune responses were higher after chemotherapy compared to responses before surgery ($P = 0.06$). Responses to recall antigens were also higher after chemotherapy compared to responses before operation ($P = 0.08$, Table 3). Before surgery, the responses against the 3 peptide pools showed no differences ($P = 0.476$). However, after chemotherapy, the response against p9-p16 was significantly higher when compared to the response against p1-p8 or p17-p24 ($P = 0.011$ and 0.009 respectively).

P53-specific responses were also detected in 46% (6/13) and 45% (5/11) of patients with benign ovarian tumors before and after surgery, respectively. After surgery, the p53-specific responses in patients with benign ovarian tumors were higher than in ovarian cancer patients ($P = 0.034$). However, responsiveness to recall antigens after surgery is also higher in patients with benign ovarian tumors than in ovarian cancer patients ($P = 0.02$, Table 3). The low p53-specific immune responses after surgery in ovarian cancer patients are probably due to an overall decrease in immune responsiveness exemplified by the decreased immune response against recall antigens MRM as well ($P = 0.05$). P53-specific immune responses could also be detected in 50% (5/10) of healthy women. P53-specific responses before surgery did not differ between ovarian cancer patients, patients with benign ovarian tumors and healthy controls ($P = 0.882$). No statistical significant correlation was found between p53-specific cellular immune responses before surgery or after chemotherapy and p53 overexpression in the tumor.

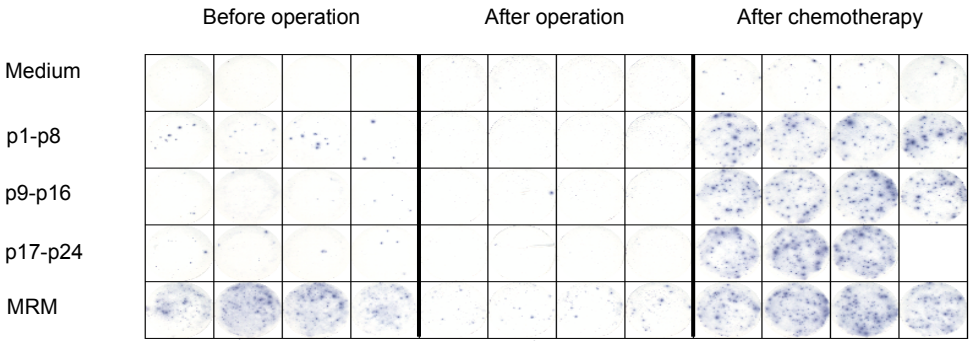

Figure 1. P53-specific responses in PBMC of an ovarian cancer patient before operation, after operation and after chemotherapy as measured by IFN-γ ELISPOT.

PBMC were stimulated *in vitro* with p53 peptides (p1-p8, p9-p16, p17-p24) or MRM for 4 days and tested by IFN-γ ELISPOT.

Table 2. Anti p53 immune responses in PBMC of ovarian cancer patients, patients with benign ovarian tumors during standard treatment and healthy controls analyzed by IFN-γ ELISPOT

Patient[1]	Before surgery			After surgery			After chemotherapy		
	p1-p8[2]	p9-p16	p17-p24	p1-p8	p9-p16	p17-p24	p1-p8	p9-p16	p17-p24
P2	-	76[3]	-	-	22	-	-	-	-
P3	-	-	-	-	-	-	29	44	33
P4	32	57	-	-	-	-	-	-	-
P7	25	-	-	-	-	-	17	27	-
P9	-	18	-	-	-	-	-	43	-
P10	na	-	na	37	-	-	na	na	na
P12	-	32	26	-	-	-	na	na	na
P13	-	-	-	-	-	-	-	19	-
P16	-	16	-	-	-	-	na	na	na
P17	-	19	-	-	-	-	35	43	25
P18	-	12	-	-	-	-	-	73	-
P19	-	-	-	-	-	-	-	33	-
P20	23	-	18	-	-	-	na	na	na
P22	-	-	16	-	-	-	18	35	-
B1	-	26	44	-	-	-			
B3	-	-	-	-	22	21			
B4	47	-	54	na	na	na			
B5	-	26	-	-	-	-			
B6	-	28	-	57	68	69			
B7	-	-	-	100	29	59			
B8	66	21	26	-	20	-			
B9	34	25	-	na	na	na			
B12	-	-	-	22	23	-			
H1	-	22	-						
H2	-	17	-						
H5	-	36	-						
H6	-	44	-						
H10	-	23	43						

[1]Twenty-three patients with primary ovarian cancer, thirteen patients with benign ovarian tumors and ten healthy controls were tested for p53-specific responses by IFN-γ ELISPOT. Only patients and controls with a p53-specific response are shown. From each patient PBMC were isolated from blood before surgery, after surgery and in case of malignancy three months after chemotherapy. [2]The pool of p53 peptides that was used to stimulate patient-derived PBMC *in vitro* for four days. [3]A patient was considered to have a p53-specific T cell response if the mean number of spots exceeded the mean number of spots of the medium+2xSD by 10 spots. Otherwise the response was considered negative (-). The number of p53-specific IFN-γ producing cells (per 10^5 PBMC) was calculated by subtracting the mean number of spots of the medium from the mean number of spots of the experimental wells. Na, PBMC were not available.

The measured p53-specific responses in ovarian cancer patients and the control groups were around threshold level. In case of these low frequencies of p53-specific T cells, the responses were more difficult to reproduce accurately. Remarkably, the responses in ovarian cancer patients were reproducible, in contrast to responses in patients with benign ovarian tumors and healthy controls. Differences in reproducibility might be a reflection of different subsets of responding T cells in patients with ovarian cancer versus patients with benign ovarian tumors and healthy controls.

In summary, low responses to the "self" antigen p53 can be detected in patients with malignant as well as in patients with benign ovarian tumors and in healthy controls.

Table 3. Responses to recall antigens (MRM) in PBMC of ovarian cancer patients and patients with benign ovarian tumors during standard treatment and healthy controls analyzed by IFN-γ ELISPOT

Patient group[1]	Before surgery	After surgery	After chemotherapy
Ovarian cancer	58%[2] (11/19)	36% (8/22)	71% (12/17)
Benign ovarian tumor	77% (10/13)	82% (9/11)	
Healthy control	90% (9/10)		

[1]PBMC were stimulated for 4 days with a memory recall mix consisting of a mixture of tetanus toxoid, *Mycobacterium tuberculosis* and Candida *albicans*. A response was considered positive if the mean number of spots exceeded the mean number of spots in the medium + 2 x SD by 10 spots. [2]Percentage of patients with a positive response to MRM.

Naïve versus memory p53-specific responses

Because p53-specific responses were found in ovarian cancer patients, as well as in patients with benign ovarian tumors and healthy controls, we further characterized these immune responses by sorting PBMC for $CD45RA^+$ and $CD45RO^+$ (memory T cells). From 7 ovarian cancer patients (P2, P3, P9, P10, P17, P18, P41) and 4 patients with benign ovarian tumors (B1, B7, B8, B12), whole PBMC, $CD45RA^+$ and $CD45RO^+$ T cell populations were analyzed for p53-specificity in the IFN-γ ELISPOT (Figure 2). Most interestingly, in 5 out of 7 ovarian cancer patients, the p53-specific immune responses were mixed memory and $CD45RA^+$ T cell responses. In ovarian cancer patients P3 and P41 responses were only found in the $CD45RA^+$ T cell fraction or in the memory T cell fraction, respectively. No p53-specific T cell reactivity was detected in the memory T cell population of patients with benign tumors, albeit reactivity was found in the $CD45RA^+$ T cell population. In the absence of p53-specific memory T cells, immune reactivity against p53 in the $CD45RA^+$ fraction is likely to represent a response by naïve T cells. As expected, MRM responses were detected in both $CD45RA^+$ and $CD45RO^+$ T cell populations.

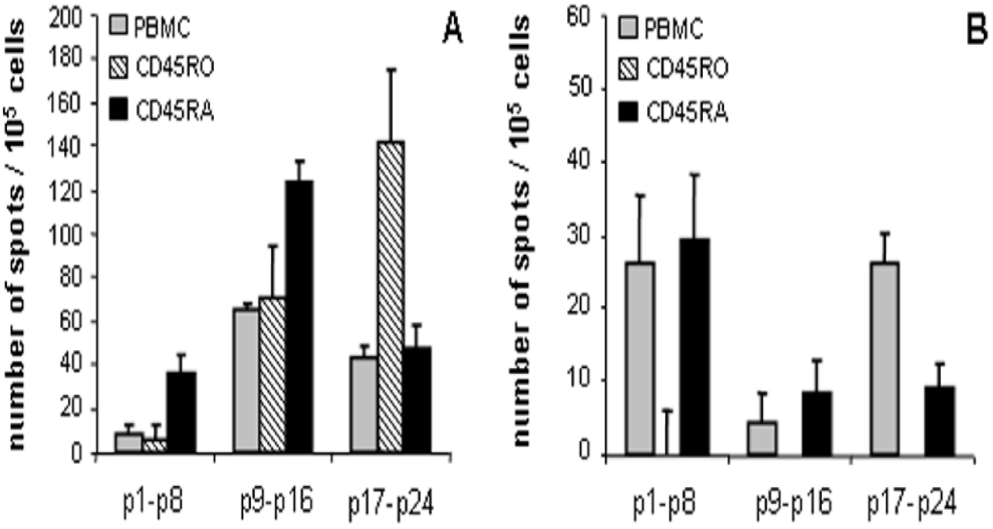

Figure 2. **P53-specific T cell responses in whole PBMC, CD45RA$^+$ PBMC (naïve T cells) and CD45RA$^-$ PBMC (memory T cells) of A. an ovarian cancer patient and B. a patient with a benign ovarian tumor.**

CD45RA$^+$ cells were isolated with magnetic beads. CD45RA$^+$ and CD45RA$^-$ PBMC populations were stimulated with p53 peptides and tested by IFN-γ ELISPOT. The number of p53-specific IFN-γ producing cells (per 10^5 PBMC) was calculated by subtracting the mean number of spots of the medium from the mean number of spots of the experimental wells.

P53-specific T cell reactivity in ovarian cancer patients analyzed by p53-specific proliferation assay

P53-specific responses in PBMC were also analyzed using a proliferation assay according to a previously established optimized protocol (38). P53-specific responses were detected in 32% (7/22) of the ovarian cancer patients, in 33% (3/9) of the patients with benign ovarian tumors and in 50% (4/8) of the healthy women (Table 4).

PBMC of responders in the proliferation assay were also tested against 12 p53 peptide pools containing 2 of the overlapping p53 peptides each (Figure 3). The responses against 2 peptides per pool were not determined for patient 25 because of shortage of PBMC. As a control, patients without a p53-specific response against one of the 3 pools (containing 8 peptides) were also tested. As expected, none of them showed an immune response against the pools containing 2 peptides (data not shown). P53-specific responses were found to 8 of the 12 peptide pools. Most responses were found against p7-p8 and p17-p18.

Four out of 7 patients reacted against p7-p8 and 3 out of 7 patients reacted against p17-p18. No responses were found against p5-p6, p11-p12, p15-p16 and p23-p24. Patients 31 and 45 showed a positive response to only one of the 12 peptide pools (p7-p8 and p17-p18, respectively). In all other patients, p53-specific responses were directed against multiple epitopes.

Table 4. P53-specific immune responses in freshly isolated PBMC of ovarian cancer patients, patients with benign ovarian tumors and healthy controls analyzed by proliferation assay

Patient[1]	p1-p8[2]		p9-p16		p17-p24	
	CPM	SI	CPM	SI	CPM	SI
P25	-	-	-	-	1621[3]	6.7
P31	22229	3.9	-	-	-	-
P32	7937	5.9	7969	7	-	-
P40	4537	9.6	12405	4.3	11655	5.9
P41	-	-	-	-	2961	5.7
P44	13636	5	4385	3.2	8538	6.9
P45	-	-	-	-	8199	4.3
B10	-	-	1126	3.8	-	-
B12	-	-	2189	3.5	-	-
B19	12238	7.9	6126	8.8	7258	4.5
H1	-	-	10811	3.6	-	-
H4	8395	4.3	-	-	-	-
H7	-	-	3912	3.9	-	-
H8	9665	6.1	12447	3.1	11453	4.2

[1]Twenty-two patients with primary ovarian cancer, 9 patients with benign ovarian tumors and 8 healthy controls were tested for p53-specific responses by proliferation assay. Only patients and healthy controls with a p53-specific immune response are depicted in this table. From each patient PBMC were isolated from blood before surgery. [2]The pool of p53 peptides used to stimulate patient-derived PBMC *in vitro* for 9 days. For the following 10 days PBMC were nonspecifically stimulated with irradiated autologous PBMC and PHA. Proliferation was measured, after 2 days of stimulation with p53 peptides, by ^{3}H-thymidine incorporation. [3]A patient was considered to have a p53-specific T cell response if the mean number of counts per min (CPM) exceeded 1,000 and if counts of the experimental wells exceeded the counts of the medium control at least 3 times (SI≥3). Otherwise, the response was considered negative (-). Na, PBMC were not available.

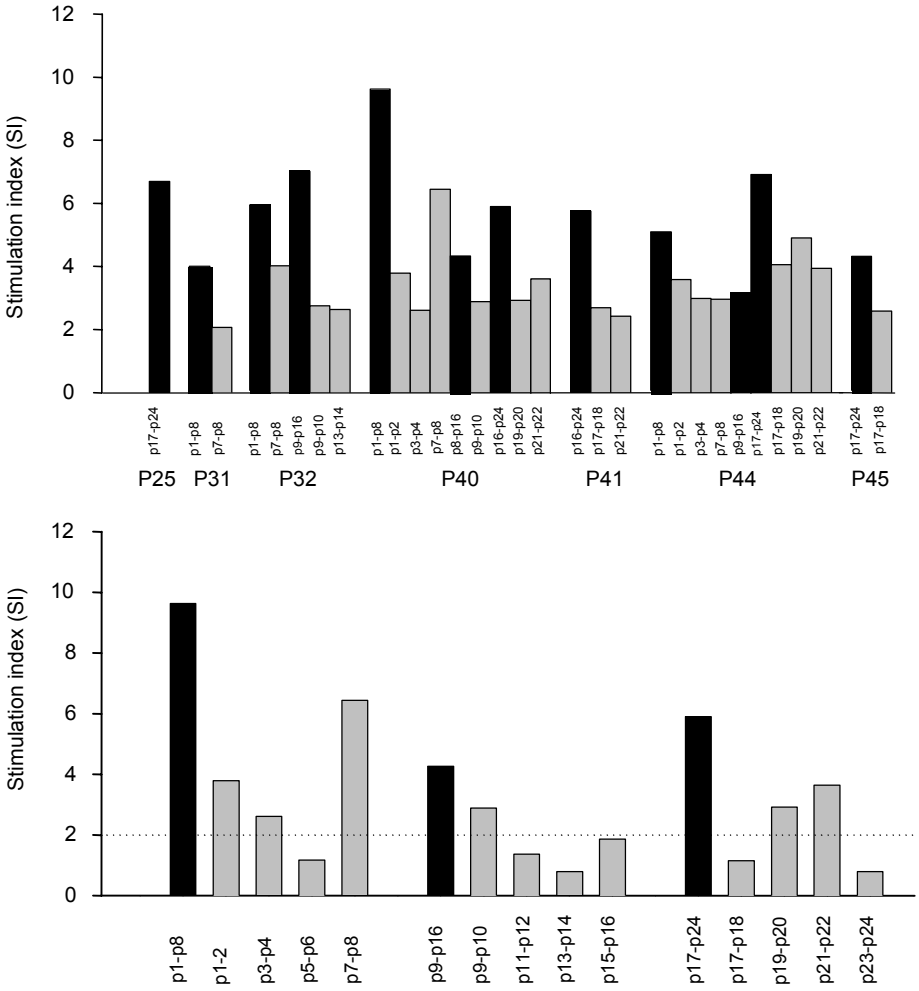

Figure 3. P53-specific T cell reactivity is directed towards multiple epitopes in ovarian cancer patients as measured by proliferation assay.

A. P53-specific responses in ovarian cancer patients. Only patients (P25, P31, P32, P40, P41, P44, P45) and peptide pools with a positive p53 response are shown. **B.** Responses to the p53 peptide pools in patient 40. The responses in the PBMC against the 3 pools of 8 overlapping p53 peptides (black bars; p1-p8, p9-p16, p17-p24) and the 12 pools of 2 overlapping p53 peptides (gray bars; p1-p2 to p23-p24) are shown. Cultures stimulated with the pools containing 8 peptides were considered positive when the SI of antigen-induced proliferation was ≥3. Cultures stimulated with the pools containing 2 peptides were considered positive when SI ≥2.

Table 5. P53-specific immune responses in freshly isolated PBMC, TIL and lymphocytes from the lymph node of ovarian cancer patients analysed by proliferation assay

Patients[1]			P34	P37	P43	P44	P45
PBMC	p1-p8[2]	CPM	-	-	-	13636[3]	-
		SI	-	-	-	5	-
	p9-p16	CPM	-	-	-	4385	-
		SI	-	-	-	3.2	-
	p17-p24	CPM	-	-	-	8,538	8199
		SI	-	-	-	6.9	4.3
TIL	p1-p8	CPM	na	2182	-	na	-
		SI	na	3.0	-	na	-
	p9-p16	CPM	na	-	-	na	3700
		SI	na	-	-	na	11.2
	p17-p24	CPM	na	-	-	na	-
		SI	na	-	-	na	-
Lymph node	p1-p8	CPM	6932	na	2251	-	-
		SI	4.3	na	4.3	-	-
	p9-p16	CPM	7095	na	7949	-	-
		SI	5.9	na	20	-	-
	p17-p24	CPM	5175	na	5312	-	6311
		SI	4.2	na	4.8	-	4.1

[1]PBMC, tumor infiltrating lymphocytes (TIL) and lymphocytes from the lymph node of 5 ovarian cancer patients were tested for p53-specific responses by proliferation assay. [2]The pool of p53 peptides used to stimulate patient-derived PBMC, TIL or lymphocytes from the lymph node *in vitro* for 9 days. For the following 10 days cells were nonspecifically stimulated with irradiated autologous PBMC and PHA. Proliferation was measured, after 2 days of stimulation with p53 peptides, by ^{3}H-thymidine incorporation. [3]A patient was considered to have a p53-specific T cell response if the mean number of counts per min (CPM) exceeded 1,000 and if counts of the experimental wells exceeded the counts of the medium control at least 3 times (SI≥3). Otherwise, the response was considered negative (-). Na, not available.

Cytokine profiles

To determine the polarization of the p53-specific T cell responses, Th1 and Th2 cytokines (IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α) were measured by cytokine bead array. Cytokines were measured in the supernatant of p53-specific positive cultures, detected by proliferation assay. PBMC of P31 produced IL-4, IL-5 and IFN-γ upon stimulation with p53 peptides p1-p8. Only IL-10 was detected in the cultures of P32 responding to peptides p1-p8 and p9-p16. In the cultures of P40 with a p53-specific response, IL-2, IL-4, IL-5, IL-10 and IFN-γ were detected. P44 produced IL-10 and TNF-α and P45 produced IL-4 and IL-5

(all data not shown). In the p53-responsive culture supernatants of P25 no Th1/Th2 cytokines could be detected.
In summary, 2 patients produced only Th2 cytokines and 3 patients showed a mixed Th1/Th2 response upon stimulation with p53 peptides.

P53-specific responses in tumor and lymph node

Tumor and lymph nodes were obtained from respectively 3 (P37, P43, P45) and 4 (P34, P43, P44, P45) ovarian cancer patients. Tumor infiltrating lymphocytes (TIL) and lymphocytes from the lymph node were isolated and analyzed for p53-specific immune responses in a proliferation assay (Table 5). A p53-specific response was detected in the TIL of P37 and P45. The lymphocytes from the lymph nodes of patients P34, P43 and P45 showed a p53-specific immune response. Yet, only in the PBMC of P45 a p53-specific response to the corresponding p53 peptide pool was detected. The p53-specific proliferation in the lymphocytes of the lymph node and in the PBMC of P45 was of the same magnitude. The p53-specific response in the TIL of P45 was detected against another p53 peptide pool than the response against the PBMC and lymphocytes of the lymph node. In summary, p53-specific responses can be detected in the tumor and lymph nodes of ovarian cancer patients. However, responses in the PBMC, lymph node or at the tumor side do not necessarily correspond with each other.

Discussion

Improved survival of ovarian cancer patients with intratumoral T cells, and in more detail high $CD8^+$/Treg ratios, suggests that the immune system plays a favorable role in controlling the tumor (2;39). Furthermore, the presence of p53-specific antibodies in advanced stage ovarian cancer patients correlates with improved survival, indicating that immunity against p53 has a favorable effect on survival (34). In ovarian cancer patients, T cell responses against undefined tumor-antigens could be found at low but detectablelevels in PBMC, when stimulated with autologous tumor cells (40). In the present study an extensive analysis of the p53-specific immune response in ovarian cancer patients during standard treatment was performed. These data are of major importance for the design of an effective p53-based vaccination strategy.
We observed p53-specific T cell responses in half of the ovarian cancer patients, but also in half of the patients with a benign tumor and healthy controls as measured by IFN-γ ELISPOT. In ovarian cancer patients both naïve and memory p53-specific T cells were

present, strongly suggesting that cancer-derived p53 can effectively be presented and activate circulating p53-specific T cells to become memory T cells. In contrast, in patients with benign ovarian tumors only naïve p53-specific responses were detected. The responses we detected in patients with benign ovarian tumors and healthy controls and partly also in ovarian cancer patients are most likely the result of *in vitro* priming of naïve $CD45RA^+$ T cells. As expected for a self-antigen, the p53-specific responses were low and around threshold level, which indicates the presence of only low avidity T cells (41). P53-specific proliferative Th-immunity has been observed in the majority of patients with primary colorectal cancer (38;42). However, in these patients the responses were also low, comparable to the responses we measured in ovarian cancer patients.

Our data indicate that the middle part of the p53 protein is the most immunogenic part, comparable to the observation in colon cancer patients (42). Moreover, the middle part of the p53 protein contains most of the known MHC class I and II epitopes (43). Finding p53 overexpression in 65% of the malignant ovarian tumors and p53-specific antibodies in 9% of ovarian cancer patients is in accordance with the literature (14;44;45). The absence of correlation between p53-specific immune responses before surgery and p53 overexpression in the tumor indicates that overexpression of p53 in the tumor is not a prerequisite for induction of p53-specific responses.

PBMC cultures of 6 out of 7 patients displaying a p53 responses in the proliferation assay were analyzed for Th1/Th2 cytokines. In most patients (5/6) Th2 cytokines were detected. Yet, in half of the patients (3/6), the PBMC also displayed a Th1 profile upon activation with p53 peptides. Taken together, the polarization state of the p53-specific T cells is directed more towards humoral than towards cell-mediated immunity. As the p53 responses as observed in this study were predominantly Th2 type, one could, for vaccination purposes aiming at inducing a cellular immune response, consider to use an adjuvant that skews the response towards a Th1 response.

The low postoperative p53-specific responses in ovarian cancer patients compared to patients with benign ovarian tumors might be explained by the significant higher blood loss and duration of surgery for ovarian cancer patients. In addition, postoperative immunosuppression was hypothesized to be due to an increase in immunosuppressive cytokines and proteins, a decrease in Th and CTL populations and induction of Treg cells in esophageal cancer (46). Therefore, vaccination should not be performed too soon after surgery.

P53-specific responses were higher after standard treatment (surgery followed by chemotherapy) than before treatment. The release of high amounts of p53 in the circulation

due to apoptotic cell death induced by chemotherapy might be an explanation. However, there was no correlation between high p53 expression in the tumor and p53-specific responses after standard treatment. Chemotherapy-induced apoptosis was found to enhance cross-presentation and cellular anti-tumor immunity (47). Furthermore, immunization after chemotherapy with a tumor-antigen resulted in a stronger anti-tumor response compared to immunization without chemotherapy (48;49). It has been shown that sensitization of tumor cells by chemotherapeutic drugs makes them more susceptible for killing by the immune system and may enhance immunotherapy (50). Increase of the immune responses after chemotherapy could also be caused by chemotherapy-induced down-modulation of regulatory T cell frequency and function (51). Based on these results, we consider that vaccination should be initiated after chemotherapy. From a clinical point of view, vaccination after chemotherapy is also logical as in many patients only small residual disease is left after standard treatment.

Several immunotherapeutic clinical trials targeting p53 have already been performed (52-55). In these studies, advanced stage cancer patients were vaccinated with either dendritic cells transduced with an adenoviral vector encoding the full-length wt.p53 gene, with recombinant canarypoxvirus (ALVAC) encoding wt.p53 or with autologous dendritic cells loaded with wild-type and modified p53 peptides. In all clinical trials, p53-specific immune responses were significantly enhanced after vaccination in part of the patients. However, clinical responses were hardly seen, probably because the induced magnitude and polarization of the T cells was not sufficient to provoke regression of high tumor burdens. Absence of clinical response may also be due to the inclusion of heavily pretreated patients with advanced stage disease.

In our study, p53-specific responses were also detected in tumor infiltrating lymphocytes and T cells of the lymph nodes. However, immune suppression by the tumor microenvironment can diminish effective immune responses against the tumor (56;57). P53-based vaccination should aim at boosting the small repertoire of p53-specific memory T cells already present in patients. Therefore, p53-specific T cells should be skewed towards a Th1 profile. The detection of p53-specific T cell responses by stimulation of PBMC with long overlapping peptides, indicates that the immune system is capable of mounting a T cell response to physiological amounts of tumor-derived p53 presented by APC, albeit that this T cell response is weak and Th2-polarized. The existence of a p53-specific T cell repertoire supports the rationale of using p53 long peptides in vaccination strategies aiming at the induction of p53-specific Th1/CTL immunity (58). We anticipate that the induced p53-specific responses can overcome the suppressive effect of the tumor

on the immune system and prevent outgrowth of recurrent disease after standard treatment.

In summary, previous murine research showed that p53 may be a suitable target antigen for therapeutic vaccination against tumors (17;18). We demonstrated the presence of modest p53-specific memory T cell responses in ovarian cancer patients in a longitudinal study. Therefore, p53 may be an attractive target for immunotherapy of ovarian cancer. The highest p53 responses occur after primary treatment, a time point at which most ovarian cancer patients have no or only small residual disease. Thus, p53 may be a suitable target for immunotherapy of ovarian cancer in an adjuvant setting after primary treatment.

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